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SOLUBLE LIGNIN–CARBOHYDRATE COMPLEXES FROM SHEEP RUMEN FLUID: THEIR COMPOSITION AND STRUCTURAL FEATURES

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ABSTRACT

Lignin–carbohydrate complexes, isolated from cell-free rumen fluid of sheep, consisted of polyphenolic material in association with carbohydrate (5.5%) and protein (1.8%). No separation of carbohydrate from the other components could be effected. The complexes showed a bimodal distribution of molecular size with the higher-molecular-weight fraction richer in carbohydrate. Methylation analysis indicated a wide range of linkages and a high proportion of terminal sugars. Reducing sugars were found in the complexes, particularly in the low-molecular-weight fraction (~25%), suggesting that ether linkages to the phenolics were also present.

INTRODUCTION

Comparatively little is known of the relationship between lignin and the other components of plant cell walls, despite the importance of such a relationship in any consideration of the utilization of plants by ruminants. Progressive lignification occurs with increasing maturity of the plant and is accompanied by a decline in the breakdown of cell-wall material by rumen micro-organisms[1] possibly due to physical shielding of the digestible cell-wall polysaccharides by increasing amounts of insoluble lignin, inhibition of enzyme activity by increasing numbers of phenolic residues in lignin, and increases in the number of bonds between lignin and the other cell-wall components[2].

Although there has been no direct proof of the existence of chemical bonds between lignin and carbohydrate, the weight of indirect evidence makes it difficult to reject such a hypothesis[3,4]. Most of the evidence has come from investigations on complexes isolated from wood, and various types of covalent linkage have been postulated[5]. The occurrence of similar linkages in the cell walls of grass and other plant materials of the animal diet has been investigated much less extensively, but it is generally considered that, for wood, some of the lignin is chemically bound to polysaccharides of the cell wall and the remainder is attached to other components.

Lignin in plant material was formerly considered to be undigested by

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ruminants and was often used as a marker in digestibility studies. Recently, Gaillard and Richards[6] found, in cell-free rumen fluid from cattle fed on a grass diet, soluble polymers that consisted of polyphenolic material closely associated with carbohydrate. The polyphenolic material must have been a precursor of, or originated from, lignin. The inability to separate polyphenol from carbohydrate by gel filtration was strongly suggestive of the existence of covalent linkages between them. Later work provided further indirect evidence for the existence of such linkages and showed that the complexes became insoluble on passing to regions of low pH in the intestinal tract, appearing as solid matter in the faeces[7]. There was no evidence of lignin from the plant being metabolised to a significant extent, but the solubilisation of a large proportion in the rumen invalidated the use of lignin as a marker in digestibility studies.
studies.

In cell-free rumen fluid from sheep fed on a diet of dried grass, similar polyphenolic material is found in close association with carbohydrate and protein[8]. Methylation analysis has been applied to the carbohydrates present in the soluble polymers and in the intact cell walls of the grass feed. The results indicate the presence of much more complex and ramified structures in sheep rumen liquor complexes (RLC) than those described[9] for bovine rumen liquor.

RESULTS

Analysis of the complexes. — High-speed centrifugation of rumen fluid gave a cell-free supernatant solution which contained 12 mg of neutral sugar (determined as glucose) per 100 mL. On dialysis, 24–50% of this carbohydrate was removed. The freeze-dried material, which was dark-brown in colour and of limited solubility (< 1 g in 100 mL of water at room temparture), contained carbohydrate (as glucose) 5.1%, uronic acid 1.4% acetyl 0.24%, and nitrogen 4.7%.

The nitrogen content suggested that most of the sample was protein, and the remainder appeared to be polyphenolic material. The nature of this material precluded its quantitation by methods established for lignin. The Klasen method resulted in considerable loss of lignin-derived material due to dissolution during analysis. In the acetyl bromide procedure, absorption values vary with changes in lignin structure, rendering this method unsatisfactory without an appropriate standard. There was also interference by proteins and peptides. Folin's reagent was used for monitoring total phenolics in the fractions. This measurement included protein and any flavonoids and tannins as well as phenylpropanes (lignins).

Detailed analysis of the freeze-dried material revealed seven sugars (Table I). Glucose was always the most abundant sugar, with rhamnose and galactose in considerable amount, and smaller quantities of fucose, arabinose, xylose, and mannose. Analysis of the original dried-grass feed and the lignin–carbohydrate complexes (LCC) extracted with 1,4-dioxane from dried grass showed that each contained all of the sugars present in the soluble rumen complexes although the propor-

TABLE I

COMPOSITION OF RUMEN LIQUOR COMPLEXES AND RELATED MATERIAL

	Percentage of total neutral sugar		
	RLC[a]	*LCC*	*Dried grass*
Rhamnose	19.0	0.3	1.0
Fucose	9.7	0.2	0.7
Arabinose	9.5	10.0	8.9
Xylose	7.0	13.0	24.0
Mannose	6.8	3.3	2.2
Galactose	17.1	8.8	4.2
Glucose	29.2	64.0	58.7
Neutral sugar (%)	3.44	36.61	35.41
Uronic acid (%)	1.9	2.28	2.76
Acetyl (%)	1.08	0.77	0.99
Nitrogen (%)	4.33	1.75	2.49

[a]RLC, rumen liquor complexes; LCC, lignin-carbohydrate complexes extracted from dried grass.

tions were very different (Table I).

Fractionation of rumen liquor. — Dialysed freeze-dried RLC could be fractionated on BioGel P-200, Sephacryl S-200, Sephadex G-100, and Sephadex G-50 into two heterogeneous components which differed considerably in molecular size. A typical separation is shown in Fig. 1. In each fraction, elution of carbohydrate and polyphenol coincided, but, whereas the recovery of carbohydrate was usually quantitative, a proportion of the polyphenolic material was adsorbed irreversibly. The adsorbed material was associated with the fraction of lower molecular weight and may have included flavonoids which would be detected by the Folin assay. Fractionations on DEAE-Sephadex and DEAE-Sepharose were unsatisfactory due to poor recoveries of the carbohydrate and polyphenols.

The complexes always showed a bimodal distribution of molecular size. The molecular weight of fraction A (Fig. 1) was probably $>100{,}000$ since it appeared in the void volume of columns of BioGel P-200 and Sephadex G-100. It was not possible to determine the average molecular weight of fraction B since there was evidence of interaction of the complexes and the gels. Such interaction has been noted previously[10]. Even on BioGel P-4, with an exclusion mol. wt. limit of ~ 4000, the bulk of the fraction was eluted in a volume equal to that required to elute the free hexoses.

Separation into two fractions on the basis of molecular size was more readily achieved by the passage of dialysed cell-free RLC through an Amicon ultrafiltration cell fitted with a PM-10 membrane (approximate mol. wt. cut-off 10,000). The two fractions obtained, containing material of high (I) and low (II) molecular weight, were similar to those obtained by gel filtration, and, with this method, reasonably

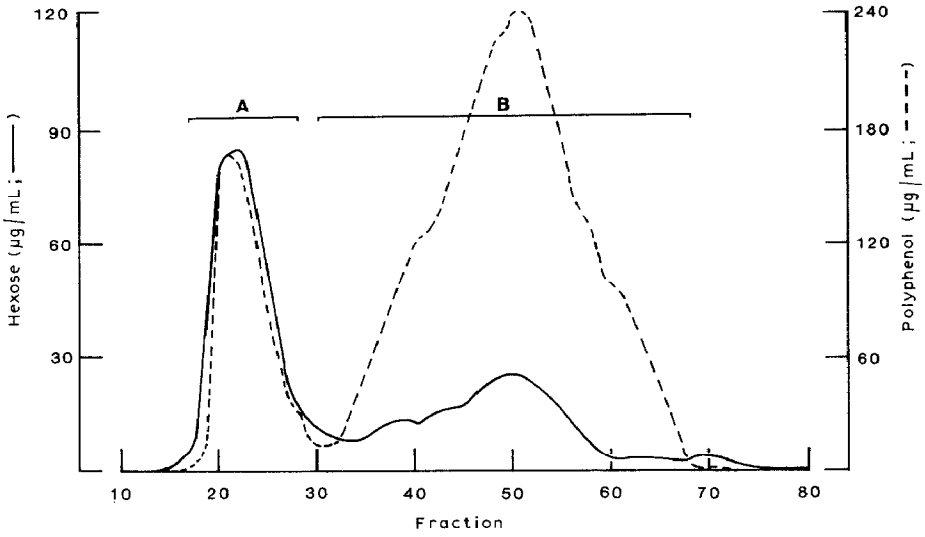

Fig. 1. Fractionation of RLC (0.3 g) on Sephadex G50 (see Experimental): ————, carbohydrate; ------, polyphenol/protein.

large volumes of rumen fluid could be fractionated fairly rapidly. Each fraction contained the range of sugars found in the origial rumen liquor, but fraction I was considerably richer in carbohydrate, and the proportions of the sugars varied between the two fractions (Table II).

TABLE II

COMPOSITION OF FRACTIONS FROM RUMEN LIQUOR COMPLEXES

	Percentage of total neutral sugar			
	I[a]	*II*	*A*	*B*
Rhamnose	17.4	11.1	17.6	8.3
Fucose	10.4	5.4	12.8	4.9
Arabinose	5.8	18.3	3.5	14.7
Xylose	6.5	8.5	3.1	9.0
Mannose	8.0	5.7	10.1	8.3
Galactose	17.5	13.5	22.8	17.9
Glucose	34.4	37.5	30.1	36.9
Neutral sugar (%)	5.04	1.67	8.21	3.07
Uronic acid (%)	2.9	0.42	1.85	0.23
Acetyl (%)	1.43	0.58	1.58	0.48
Nitrogen (%)	6.02	4.02	4.59	4.03
Protein N (%)			3.18	1.08
Non-protein N (%)			1.41	2.95

[a]For description of fractions, see text.

The high content of nitrogen of each fraction raised the question as to whether the protein or peptide present was separable from the other components. Solutions of RLC were brought to various degrees of saturation with ammonium sulphate. In each of the resulting precipitates, the recovery of carbohydrate closely paralleled that of polyphenolic/protein material. The yield of each component in the precipitate also paralleled the weight yield, suggesting that all the components were interlinked and that fractionation was based mainly on molecular size. This view was borne out by the relatively constant percentage of nitrogen in each precipitate. A similar lack of separation into individual components was observed in fractions precipitated with increasing concentrations of ethanol. Gel filtration on Sephadex equilibrated in 8M urea gave no separation of protein. Amino acids were released from aqueous solutions of the complexes by Pronase and pepsin, but only to a limited extent, and the residual material still contained most of the nitrogen.

When solutions of dialysed samples of RLC in 0.1M acetate buffer (pH 6.0) were dialysed, there was a loss of carbohydrate and polyphenol/protein. Losses were particularly great for fraction II, often amounting to 30% of the original content. Despite losses, the relative proportions of the various sugars remained constant. Other buffers also caused disaggregation of the complexes without separation of the components.

On free-flow electrophoresis of the complexes in Tris buffer, some enrichment of carbohydrate in particular fractions was obtained, but the ratio of sugars remained constant and no evidence was obtained of unlinked protein.

Partition of complexes with phenol. — Proteins and glycoproteins closely associated with other material such as carbohydrate can be separated by extraction of a buffered solution of the mixture with hot phenol. Fractions I and II were partitioned between borate buffer (pH 8.0) and hot phenol (Table III). The major part (~80%) of the carbohydrate of each fraction remained in the borate buffer phase. Phenol extracted a considerable amount of nitrogenous material from each fraction which was accompanied by an equal proportion of polyphenolic material.

TABLE III

DISTRIBUTION OF COMPONENTS OF FRACTIONS FROM RUMEN LIQUOR COMPLEXES AFTER PHENOL EXTRACTION

	Percentage of total			
	Fraction I		*Fraction II*	
	B[a]	*P*	*B*	*P*
Carbohydrate	79	9	79	15
Polyphenol[b]	45	53	70	30
Nitrogen	42	42	64	30

[a]B, Borate buffer phase; P, phenol phase. [b]Polyphenol includes both polyphenolic and protein material.

Each of the partition procedures gave small amounts of insoluble material containing all three components at the interface of the phases. Successive extractions of the borate buffer phases failed to remove more polyphenolic or nitrogen-containing material.

Dialysis of the extracted borate-buffer phase containing fraction I resulted in little loss of polymeric material, but the borate phase from fraction II lost ~38% of its carbohydrate, nitrogenous, and polyphenolic components on dialysis. The similarity in behaviour of all three components confirmed their close association. Dialysis of the phenol extracts precipitated the greater part of the contents of polyphenols and nitrogen. Detailed comparison of the sugar composition of the borate and phenol phases from each fraction showed that, although only small amounts of all the sugars were extracted with phenol, there was relatively greater extraction of arabinose and xylose (Table IV). This finding might reflect differences in the composition or structure of complexes containing these sugars. In each of the fractions, the values of protein nitrogen based on the content of amino acids accounted for only 30–40% of the total nitrogen in the fraction (see below).

Extractability of material by phenol appeared to be based on the absence of attached carbohydrate, even though this comprised only a small proportion of each fraction. The results again suggested that there was no loosely bound protein in either fraction and that each consisted of two groups of complexes, one containing polyphenolic, nitrogenous, and carbohydrate components in close association, and the other (extractable by phenol) containing polyphenolic and nitrogenous components with little or no carbohydrate.

Origin of nitrogen in the complexes. — Determinations of protein on the fractions were complicated by doubt as to the origin of some of the nitrogen. The content of nitrogen of dialysed, freeze-dried rumen liquor indicated that, if all the nitrogen originated from protein, then ~30% of the material consisted of protein or

TABLE IV

SUGAR COMPOSITION OF FRACTIONS FROM RUMEN LIQUOR COMPLEXES AFTER PHENOL EXTRACTION

	Percentage of total neutral sugars					
	Fraction I			*Fraction II*		
	O[a]	*B*	*P*	*O*	*B*	*P*
Rhamnose } Fucose }	29.7	31.2	29.7	16.1	19.6	17.5
Arabinose	6.9	4.4	8.9	23.2	17.6	25.0
Xylose	4.6	3.4	4.8	8.9	7.9	10.0
Mannose	7.5	6.9	9.9	4.5	4.9	2.5
Galactose	19.7	20.4	21.6	10.7	12.8	10.0
Glucose	31.4	32.8	26.9	33.9	35.3	32.5

[a]O, Original fraction; B, borate buffer phase; P, phenol phase.

TABLE V

COMPARISON OF TOTAL NITROGEN CONTENT AND NITROGEN CONTENT CALCULATED FROM AMINO ACID COMPOSITION

Sample[a]	*Total N (%) (from Kjeldahl)*	*Protein N (%) (from amino acids)*	*Percentage as protein N*
RLC	4.33	1.77	40.9
Fraction I	5.50	3.04	55.3
Fraction II	4.0	1.32	33.0
LCC	1.75	0.96	54.7
Dried grass	2.49	1.22	49.0
Ovalbumin	13.42[b]	12.00	89.4
Ovamucoid	11.54[b]	9.92	86.0

[a]RLC, rumen liquor complexes; LCC, lignin–carbohydrate complexes extracted from dried grass.
[b]Nitrogen contribution of hexosamine has been deducted.

peptide. However, calculation of the content of nitrogen from amino acid analysis accounted for only 40% of the total nitrogen as determined by the Kjeldahl method. Samples of Fractions I and II, dried grass, and lignin–carbohydrate complexes (LCC) extracted from dried grass each gave a low recovery of nitrogen based on their amino acid analyses (Table V). As controls, the glycoproteins ovalbumin and ovomucoid were analysed using the same conditions. The protein nitrogen value for each glycoprotein was in agreement with the total nitrogen value. Since the two plant samples that had very high contents of carbohydrate (dried grass and LCC) gave recoveries of nitrogen as protein which were similar to those obtained from the other plant fractions, it seemed likely that the low recoveries of nitrogen from plant materials were not due to interaction with carbohydrate products, but were related in some way to the polyphenolic component.

During hydrolysis for the determination of amino acids, each of the plant products gave a dark-brown insoluble residue consisting mainly of polymerised polyphenols. Each residue contained nitrogen, the proportion being greater in samples with a high content of polyphenol. However, there was insufficient nitrogen to account for the 40–60% of nitrogen which was not identified as protein (Table VI). Protein nitrogen accounted for only 46–65% of the total nitrogen in the hydrolysis supernatant solution, corresponding to 33–58% of the nitrogen in the original sample (Table VI). Thus, it seemed probable that the nitrogen remaining in solution was incorporated in some part of the polyphenol molecule.

Action of dilute acid. — Treatment of RLC with 0.05M H_2SO_4 at room temperature gave a precipitate that contained 20–35% of the total nitrogen, neutral sugar, and polyphenol–protein. Protein nitrogen was distributed equally between precipitate and supernatant solution, but the latter was 4.5 times richer in non-protein nitrogen than the former. Dialysis of the supernatant solution resulted in the loss of 33–45% of its non-protein nitrogen, protein nitrogen, and polyphenol, with

TABLE VI

DISTRIBUTION OF NITROGEN AFTER HYDROLYSIS OF COMPLEXES

	Percentage of total N	*Percentage as protein N (calc. from amino acids)*	*Percentage of total N as protein N*
Fraction I			
Supernatant	81.1	68.2	55.3
Residue	18.3		
Fraction II			
Supernatant	71.8	46.0	33.0
Residue	22.5		
LCC			
Supernatant	88.5	65.3	57.8
Residue	6.4		

somewhat smaller losses (20%) of sugar. Compared with RLC, the precipitate was richer in arabinose and xylose. The increase in the total sugar content of the supernatant solution compared to that of the original RLC reflected the relatively greater loss of polyphenolic components, indicating that there may have been some splitting of acid-labile linkages between polyphenol units.

A similar distribution of nitrogen was obtained after the action of dilute acid on an alkaline extract from dried grass. Dilute-alkali-extracted complexes containing >50% of the total nitrogen in the grass and treatment of this extract with acid precipitated 38% of the nitrogen present in the extract. Protein nitrogen was equally distributed between precipitate and supernatant solution, whereas 72% of the non-protein nitrogen was in the supernatant. Although the solubilised polyphenol-protein present in the extract from dried grass was obtained by the action of alkali and not by bacterial degradation as with the rumen complexes, the distribution of protein and non-protein nitrogen between precipitate and supernatant solution in each sample was similar.

Action of alkali. — Solutions of RLC and fractions I and II in M NaOH were stored overnight at room temperature and then extracted with ether. None of the extracts contained (t.l.c.) carbohydrate, but those from RLC and fraction II contained ferulic, coumaric, and vanillic acids, together with an unidentified compound which reacted as a phenolic acid. The extract from fraction I contained no free phenolic acids.

When the neutralised alkali-treated RLC was eluted from a column of Sephadex G-50 with 0.2M NaCl and the eluate was monitored for carbohydrate and polyphenol, there was a bimodal distribution with fractions having molecular sizes similar to those of fractions from untreated RLC. However, there was a transfer of both carbohydrate and polyphenol-protein from the fraction of high molecular

TABLE VII

COMPOSITION OF DELIGNIFIED FRACTIONS

	Percentage of total neutral sugar			
	I	*Delignified I*	*II*	*Delignified II*
Rhamnose	17.4	16.7	9.3	15.5
Fucose	10.4	7.5	8.8	7.9
Arabinose	5.8	5.6	18.3	14.2
Xylose	6.5	4.1	8.3	6.4
Mannose	8.0	8.1	5.4	6.9
Galactose	17.5	19.3	16.6	20.9
Glucose	34.4	35.9	33.2	28.3
Neutral sugar (%)	5.04	5.11	1.62	3.21
Uronic acid (%)	2.9		0.22	0.37

weight to that of lower molecular weight, suggesting that labile linkages between polyphenolic units had been split by alkali.

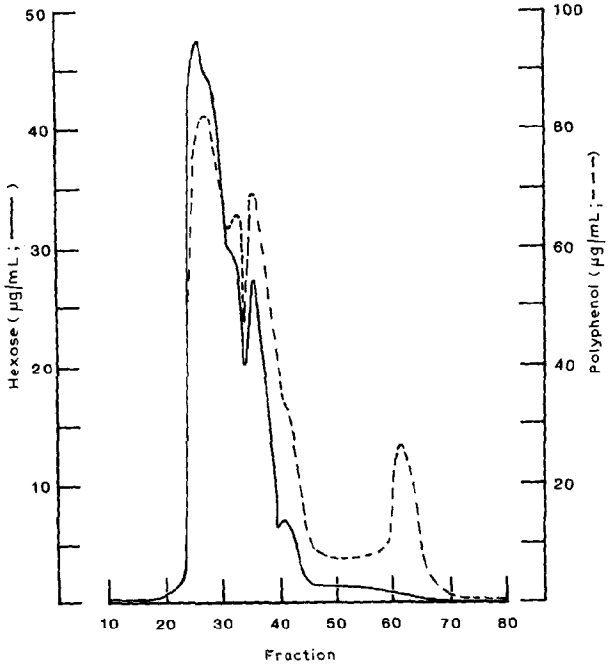

Fig. 2. Fractionation of delignified fraction I on Sephadex G25 (see text): ————, carbohydrate; ------, polyphenol/protein.

TABLE VIII

METHYLATED SUGAR ANALYSIS OF RLC AND FRACTIONS

Sugar	*Methoxyl groups*	*Percentage of sample*[a]				
		RLC	*I*	*II*	*Reduced I*	*Reduced II*
Arabinose						
	235	0.244	0.059	0.024	0.073	0.088
	23	0.099	0.021	0.011	0.027	0.036
	25	0.102	0.033	0.011	0.029	0.046
	35					
	Total	0.445 (0.539)[b]	0.113 (0.288)	0.046 (0.306)	0.129 (0.357)	0.170 (0.444)
Xylose						
	234	0.065	0.033	0.006	0.016	0.025
	23	0.172	0.044	0.012	0.047	0.029
	24[c]					
	2 } 3 }	0.096	0.038	0.008	0.030	0.021
	Total	0.333 (0.413)	0.115 (0.209)	0.026 (0.142)	0.093 (0.443)	0.075 (0.309)
Rhamnose						
	234	0.338	0.145	0.017	0.087	0.083
	23	0.314	0.139	0.008	0.043	0.040
	24	0.338	0.099	0.009	0.060	0.040
	34	0.362	0.104	0.011	0.065	0.047
	2	0.113	0.056	0.006	0.025	0.023
	3	0.098	0.078	0.005	0.023	0.015
	4	0.190	0.068	0.007	0.052	0.035
	Total	1.753 (1.824)	0.689 (0.854)	0.063 (0.186)	0.355 (0.953)	0.283 (0.292)
Fucose						
	234	0.131	0.045	0.005	0.027	0.025
	23	0.293	0.115	0.006	0.022	0.041

	24	0.359	0.125	0.014	0.056	0.049
	2	0.195	0.059	0.005	0.014	0.018
	3	0.031	0.038			
	4	0.036	0.030		0.024	0.007
	Total	1.045 (1.025)	0.412 (0.385)	0.030 (0.090)	0.143 (0.423)	0.140 (0.147)
Glucose						
	2346	0.575	0.368	0.088	0.244	0.287
	234	0.229	0.104	0.029	0.072	0.079
	236	0.646	0.291	0.037	0.155	0.116
	246	0.394	0.212	0.015	0.096	0.069
	346	0.138	0.047	0.011	0.030	0.044
	23	0.073	0.048	0.006	0.020	0.023
	24	0.027	0.037		0.002	0.010
	26	0.266	0.107	0.006	0.054	0.050
	36				0.018	0.018
	46	0.083	0.024		0.008	0.008
	2	0.033	0.013			0.004
	Total	2.464 (2.219)	1.251 (1.834)	0.197 (0.625)	0.699 (1.709)	0.708 (1.035)
Galactose						
	2346	0.413	0.120	0.026	0.101	0.084
	2356	0.048	0.010		0.011	0.013
	234	0.109	0.042	0.009	0.029	0.026
	236	0.296	0.097	0.007	0.058	0.052
	246	0.375	0.135	0.010	0.085	0.051
	256	0.073			0.012	0.004
	23	0.038	0.038			0.004
	24	0.064	0.028	0.001		0.004
	34		0.025		0.026	0.014
	36	0.056	0.016		0.008	0.012
	46	0.154	0.117	0.013	0.034	0.019
	2	0.032	0.014	0.005	0.008	0.004
	Total	1.657 (1.758)	0.642 (0.985)	0.071 (0.225)	0.372 (1.211)	0.287 (0.505)

TABLE VIII *(continued)*

Mannose						
	2346	0.338	0.099	0.009	0.060	0.040
	236	0.096	0.110	0.031	0.032	0.016
	246	0.205	0.152	0.008	0.058	0.046
	346	0.133	0.042	0.007	0.032	0.024
	24	0.035	0.022			
	26	0.102	0.045	0.005	0.033	0.030
	34	0.028	0.017		0.011	0.003
	Total	0.919 (0.756)	0.487 (0.416)	0.060 (0.095)	0.226 (0.539)	0.159 (0.297)
Total Sugar		8.633 (8.561)	3.709 (5.106)	0.493 (1.669)	2.017 (5.635)	1.822 (3.029)

[a]Quebrachitol was used as the internal standard for quantitation. [b]Figures in brackets are alditol acetate analyses of sugars, see text. [c]24-Xyl was confirmed by mass spectrometry, but chromatographed as a mixed peak with 23-Ara in SILIØC and with 2346-Gal on SP1000.

Chemical modification. — On oxidative "delignification" of the complexes with chlorine dioxide, the colour of the solutions changed from dark brown to pale yellow. Gel filtration of fraction I after this treatment showed that degradation had occurred. The bulk of the polyphenols was still accompanied by carbohydrate, but there was now an additional fraction of smaller molecular size containing polyphenol but little or no carbohydrate (Fig. 2) which was removed by dialysis. There was a small net loss of carbohydrate which must have been attached to degraded polyphenol molecules, but the carbohydrate composition of the modified fraction showed little change from that of the original (Table VII). Fraction II also gave a cleaner product after oxidation, but with larger losses of sugar on dialysis. The considerable increase in the content of carbohydrate of the freeze-dried material in spite of this loss indicated that a large proportion of the polyphenols, which form the bulk of the fraction, had been removed. The fall in the nitrogen content showed that much, although not all, of the polyphenol removed had been associated with nitrogen. As with fraction I, the relative proportions of the individual sugars were little affected. Sugar analysis and methylation analysis showed that no appreciable changes in composition or structure of the carbohydrate component had occurred.

Methylation of the complexes. — The results of methylation analysis of RLC and fractions I and II are shown in Table VIII. The identities of the components were confirmed by mass spectrometry. Minor components were identified after removal of the major peaks by preparative glc. Methylation using deuterated methyl iodide did not reveal any naturally occurring methylated neutral sugars. Reduction of methylated fraction I with lithium aluminium deuteride gave an additional product which was deuterated at C-6 and was shown by g.l.c.-m.s. to be derived from 4-linked glucuronic acid.

The recoveries of methylated sugars (Table VIII) were good for RLC and fraction I, but low for fraction II. However, the agreement with sugar composition obtained directly as alditol acetates was good in terms of relative sugar composition (Table IX). Similar results have been obtained with plant cell walls. The ratios of non-reducing terminal and branch-points and chain lengths were calculated from

TABLE IX

COMPARISON OF SUGAR COMPOSITION BY METHYLATION ANALYSIS (A) AND ALDITOL ACETATE ASSAY (B) AS PERCENTAGE OF TOTAL SUGAR

Sugar	*RLC*		*I*		*II*	
	A	*B*	*A*	*B*	*A*	*B*
Rhamnose	20.42	21.2	18.58	16.7	12.78	11.1
Fucose	12.18	11.9	11.11	7.5	6.09	5.4
Arabinose	5.18	6.2	3.05	5.6	9.33	18.3
Xylose	3.88	4.8	3.10	4.1	5.27	8.5
Mannose	10.90	8.8	13.13	8.1	12.17	5.7
Galactose	19.30	20.8	17.31	19.3	14.40	13.5
Glucose	28.71	25.9	33.73	35.9	39.96	37.5

TABLE X

TERMINAL, CHAIN, AND BRANCH-POINT RESIDUES IN METHYLATED SAMPLES

	Percentage of sample				
	RLC	*I*	*II*	*Reduced I*	*Reduced II*
Terminal	2.152	0.879	0.175	0.619	0.645
Chain	4.731	1.912	0.246	1.008	0.855
Branch point	1.815	0.945	0.077	0.398	0.330
Terminal/branch ratio	1.186	0.930	2.273	1.555	1.955
Chain length	2.198	2.175	1.406	1.628	1.326

the methylation data (Table X). The low values for chain length, particularly for fraction II, suggests that many residues are present as monosaccharides.

Identification of sugars in the complexes with free reducing groups. — Treatment of fractions I and II with sodium borohydride caused no change in the carbohydrate composition of either fraction, and confirmed that there were no alkali-labile linkages to carbohydrates. Dialysis of fraction II caused a loss of carbohydrate and polyphenol/protein due either to disaggregation or alkaline hydrolysis of linkages between phenolic units. Alditols in the reduced complexes were released by acid hydrolysis, separated from the monosaccharides, and determined quantitatively.

In fraction I, 7–8% of the total carbohydrate, consisting mainly of arabinose, xylose, rhamnose, and glucose, had free reducing groups. Some 20% of the arabinose and xylose residues had a free reducing group in the complex (Table XI). Fraction II had 20–25% of residues with reducing groups, and these were mainly arabinose, xylose, and glucose (Table XI).

The reduced fractions were examined for linkages labile to mild acid hydrolysis. During hydrolysis, insoluble dark-brown residues were formed due to polymerisation of polyphenolic material. The non-dialysable materials contained the same sugar residues as the original fractions, but were relatively poorer in arabinose and xylose. However, these sugars had not been released during hydrolysis since the insoluble residues, though of low carbohydrate content and containing all the sugars found in the original fractions, were relatively richer in arabinose and xylose (Table XI). The results may suggest the presence of acid-labile linkages in the complexes but do not enable the linkages to be identified. It is possible that, after the action of acid, any arabinose and xylose which had been attached to the complexes by glycosidic linkages remained attached by non-glycosidic linkages to other polyphenols, which then polymerised. Methylation analysis showed that the complexes contained arabinofuranoside linkages that would be expected to be acid-labile, although examples are known of furanosides in cell-wall material being inaccessible to hydrolysis. Alternatively, the splitting of acid-labile linkages between the poly-

TABLE XI

CARBOHYDRATE ANALYSES OF REDUCED FRACTIONS

Fraction I

	Percentage of total neutral sugar				
	R[a]	*E*	*(% E)*	*S*	*P*
Rhamnose	16.9	23.7	10.2	16.5	11.8
Fucose	7.5	2.7	2.6	8.5	11.3
Arabinose	6.3	18.2	21.0	4.5	9.6
Xylose	7.9	21.5	20.0	5.7	14.0
Mannose	9.6	9.4	7.2	10.7	8.8
Galactose	21.5	4.8	1.7	22.0	18.1
Glucose	30.3	19.1	4.6	32.1	26.4
Neutral sugar (%)	5.64	0.41		6.81	2.56

Fraction II

	Percentage of total neutral sugar				
	R[a]	*E*	*(% E)*	*S*	*P*
Rhamnose	9.6	4.8	10.3	12.2	2.7
Fucose	4.9	2.9	12.2	5.6	8.8
Arabinose	14.7	23.2	32.9	11.7	27.9
Xylose	10.2	18.6	37.9	9.5	16.2
Mannose	9.8	12.1	25.6	11.3	2.9
Galactose	16.7	7.8	9.7	18.7	6.8
Glucose	34.2	31.0	18.8	31.0	34.6
Neutral sugar (%)	3.03	0.63		4.39	1.30

[a]R, reduced fraction; E, reducing end groups (as alditols); (% E) = percentage of each sugar present with reducing end group; S, supernatant solution from acid hydrolysis; P, precipitate from acid hydrolysis. For details, see text.

phenols followed by polymerisation could have resulted in an increased concentration of arabinose and xylose residues attached to these polymers.

Treatment of the reduced fractions with alkali did not release any significant amount of sugar. Earlier experiments had demonstrated the release of phenolic acids from fraction II without concomitant release of carbohydrate.

Methylation analysis of reduced fraction I revealed little change compared to the original material, although the overall recovery was lower (Table VIII). Methylation analysis of reduced fraction II revealed much larger changes (Table XII). The overall change in the amount of sugar agreed with the percentage of each sugar with a reducing end group (Table XI). For each fraction, new early-running peaks were found in the chromatogram, though only for fraction II were they of an appreciable size. The new peaks were idenified by g.l.c.-m.s. as 1-*O*-methylalditols derived from

TABLE XII

COMPARISON OF ORIGINAL AND REDUCED FRACTIONS II

Sugar	*Methoxyl groups*	*Relative area*[a]		*Decrease (%)*	*Overall decrease (%)*
		II	*Reduced II*		
Arabinose					
	235	4.37	3.24	25.9	
	23	2.17	1.40	35.5	
	25	0.79	0.70	11.4	
	35				
					27.2 (32.9)[b]
Xylose					
	234	1.26	0.93	26.2	
	23	2.00	1.02	49.0	
	24				
	2	1.50	0.74	50.7	
	3				
					43.5 (37.9)
Rhamnose					
	234	3.19	2.93	8.2	
	23	2.93	3.08	−5.1	
	24				
	34	2.00	1.70	15.0	
	2	0.96	0.83	13.5	
	3	0.33	0.24	27.3	
	4	1.48	1.57	−6.1	
					5.0 (10.3)
Fucose					
	234	0.93	0.89	4.3	
	23	0.43	0.63	−46.5	
	24	2.60	1.85	28.9	
	2	0.46	0.31	32.6	
	3				
	4		0.11		
					14.2 (12.2)
Glucose					
	2346	16.88	11.83	29.9	
	234	5.02	2.89	42.4	
	236	6.44	4.20	34.8	
	246	2.66	2.44	8.3	
	346	2.09	1.65	21.1	
	23	1.00	1.00		
	24		0.13		
	26	0.97	1.73	−78.4	
	36	0.37	0.28	24.3	
	46		0.18		
	2		0.09		
					25.4 (18.8)
Galactose					
	2346	4.37	3.03	30.7	
	2356		0.20		
	234	0.63	0.92	−46.0	
	236	1.33	2.14	−60.9	
	246	1.81	1.94	−7.2	

	256		0.06		
	23		0.15		
	24	0.16	0.08	50.0	
	34		0.21		
	36		0.48		
	46	0.93	0.76	18.3	
	2	0.34	0.16	52.9	
					5.6 (9.7)
Mannose					
	2346				
	236	2.23	0.25	88.8	
	246	0.59	0.79	−33.9	
	346	1.89	1.73	8.5	
	24				
	26	0.83	1.10	−32.5	
	34		0.06		
	46				
					29.1 (25.6)

[a]Areas relative to 23-Glc = 1.00. [b]Figures in brackets are percentage change calculated from sugar analysis (Table XIV).

TABLE XIII

G.L.C.–M.S. IDENTIFICATION OF 1-*O*-METHYL SUGAR DERIVATIVES IN FRACTION II

Identification[a]	*Linkage*	*Possible corresponding methylated sugar*
1,2,3,5-pent	4	2,3-Xyl
1,2,4,5-pent	3	2,5-Ara
1,3,4,5-pent	2 (4)	
1,2,3,4-pent	5	2,3-Ara
1,4,5-pent	2,3 (3,4)	
1,2,4-pent	3,5	
1,2,5-pent	3,4 (2,3)	2-Xyl
1,2,3,5,6-hex	4	2,3,6-Glc
1,2,3,5,6-hex	4	2,3,6-Man
1,2,3,4,5-hex	6	2,3,4-Glc
1,4,5,6-hex	4,6	
1,3,5,6-hex	3,6	

[a]As 1-*O*-methylalditols derived from the reduced residues.

the reduced residues. As no standards were available, it was not possible to fully identify the peaks. However, there was a good correlation between the 1-*O*-methyl sugars which were substantially decreased on reduction (Table XII) and those identified by mass spectrometry (Table XIII).

Enzymic activity. — Freshly collected cell-free rumen liquor had appreciable xylanase activity and a low level of cellulase activity (Table XIV) but no β-D-glucosidase, β-D-galactosidase, β-D-galactosidase, β-D-xylosidase, α-D-mannosidase, or

TABLE XIV

ENZYME ACTIVITY OF RUMEN FLUID COMPLEXES

Sample	*Xylanase activity[a] (μg/mL)*	*Cellulase activity[a] (μg/mL)*
RLC	207	3.0
Dialysed RLC	79	2.8
Freeze-dried RLC	96	n.d.

[a]All activities are expressed relative to 1 mL of original cell-free rumen fluid.

β-D-glucuronidase activity. Even after prolonged storage (6 months at −20°) or freeze-drying, ~ 50% of the xylanase activity remained (Table XIV). Since the protein could not be separated from other components of rumen fluid, the enzyme must form part of the complexes present.

DISCUSSION

The soluble complexes found in the rumen fluid of sheep fed on a diet of dried ryegrass bear many resemblances to the lignin–carbohydrate complexes found in the rumen fluid of cattle fed on diets of various tropical grasses[9]. In each complex, a considerable proportion of the lignin or polyphenolic material in the plant has become solubilized as a result of enzyme activity in the rumen. However, it is unlikely that there has been a significant breakdown of lignin in the anaerobic conditions of the rumen since lignin polymers are chemically stable and are only known to be attacked by aerobic bacteria and fungi[11]. The small amount of carbohydrate associated with the soluble complexes suggests that there has been fairly complete degradation of polysaccharide in some portions of the plant with the concomitant release of polyphenolic material that had been linked to these polysaccharides.

Whether the solubility of the released polyphenol is due to the presence of closely associated components, or whether the complexes contain polyphenols that can be regarded as precursors in the formation of lignin is difficult to ascertain because of the problem of preparing comparable samples of lignin. Most methods of isolation yield products that are highly modified and include other phenolic components of the cell wall. It may well be that the soluble polyphenols originate from regions of the plant cell wall where lignification is incomplete, *i.e.*, where there are fewer cross-linkages between phenylpropanoid units and where adhesion of the polysaccharide-degrading bacteria can therefore take place more readily, leading to digestion of polysaccharide in these areas. The presence of soluble polyphenols irreversibly associated with carbohydrate is of prime importance in considering the organization of the chemical components of the plant cell wall. Since the feed has been subjected to no harsher treatment than enzymic degradation, it is unlikely that any drastic chemical modifications have occurred, and the polyphenols and their

associated carbohydrates probably represent structural fragments from the cell wall.

Although the carbohydrate content of the complexes was low, seven different sugars as well as glucuronic acid were present. Changes in the relative proportions of the sugars could be obtained by fractionation and other treatments, but it was not possible to separate any of the sugars from other components. The carbohydrate appeared to form an integral part of a range of complexes which varied principally in the molecular size of the polyphenol component and possibly in the protein content.

The complexes contained sugars (fucose and glucuronic acid) additional to those described by Neilson and Richards[9], though in earlier work[6] fucose was also found. Sugars such as fucose, rhamnose, and mannose, which form a considerable proportion of the carbohydrate in RLC, are present in very small amounts in the grass feed. Such residues may have become concentrated in the RLC due to their resistance to enzymic attack. Alternatively, they could have been derived from the extracellular capsules which surround many rumen organisms[12].

The results of methylation analysis of the various fractions showed some similarities to the findings of Neilson and Richards[9]. A major proportion of each sugar was attached glycosidically as single units to other components. However, the greater variety of methylated sugar derivatives indicated that the remainder of the sugars formed part of a much more ramified structure. The material used by Neilson and Richards[9] was a fraction that was considerably lower in carbohydrate content than the original rumen liquor.

Fraction I contained more carbohydrate than fraction II, and there were differences in the proportions of sugars and in some of the structures present. The greater number of derivatives identified on methylation analysis may also reflect the use of wall-coated capillary columns in g.l.c.[13]. Both RLC fractions contained reducing sugars, indicating attachment to the complexes by non-glycosidic linkages. In fraction II, ~20% of the sugars were linked in this way. The principal sugars involved were arabinose and xylose, one third of each having non-glycosidic linkages. Fraction I, which had a greater content of carbohydrate, had a much lower proportion of reducing sugars and these were almost entirely arabinose and xylose.

The results of methylation analysis (Table X) suggest very short carbohydrate chains, but the presence of reducing sugars indicates the presence of ether linkages between the carbohydrate units and the phenolic component. Fig. 3 shows various possible linkages. Fraction II with a higher proportion of terminal residues will have more structures of types **1–3** and, as this fraction contains ~20% of reducing sugars, it will contain significant amounts of such structures as **4** and **5**. The decrease in the amounts of non-reducing terminal residues after reduction suggests that some ether linkages to chain residues (structure **4**) are broken during methylation so that even more extended structures (*e.g.*, **6** and **7**) are possible.

Attempts to separate the carbohydrates from the complexes were not successful, so that it is not possible to assign particular sugars to the different structures although the preservation of the less substituted deoxy-sugars on reduction suggests

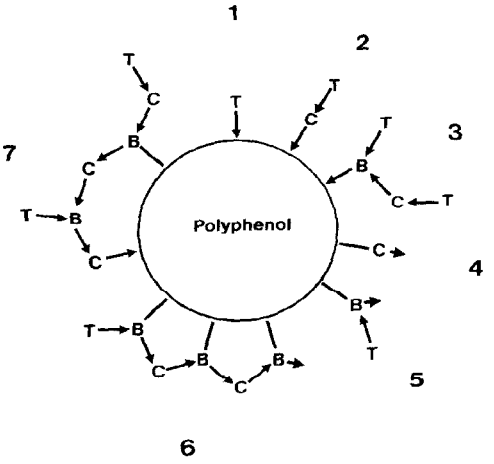

Fig. 3. Proposed structures for RLC: T, non-reducing terminal sugar residue; C, chain residue; B, branch point; →, glycosidic linkage (arrow pointing away from C-1); ————, ether linkage; ↛, free reducing group.

they are not involved in such structures as **4** and **5**.

If all the rhamnose present in the complex is of plant origin, the great variety of its methylated derivatives found in the fractions suggests that rhamnose plays an important role in cross-linkages.

All the soluble rumen complexes and LCC extracted from dried milled grass contained protein that could not be separated from other components. Partial separation of polyphenol–protein material from polyphenol was achieved, but carbohydrate is associated with both types of complex. There was no evidence for the presence in any fraction of large amounts of hydroxyproline, which has been implicated in lignin–protein linkages in the cell wall[14]. However, lignin is known to have a high affinity for protein[15]. The polyphenolic material could also include other components such as flavonoids and tannins which have a high affinity for proteins. Phenols combine reversibly with protein by hydrogen bonding, but if the phenol is oxidised, enzymically or chemically, the resulting quinones may form permanent covalent linkages to proteins. It is possible that protein or peptides that were free in the anaerobic conditions of the rumen became attached to polyphenols after exposure to aerobic conditions. The amount of protein in the complexes may have been underestimated due to losses of precipitated phenolic-protein material on hydrolysis, though this does not appear to account for all the nitrogen. Although exo-glycosidases were not present in cell-free rumen liquor, xylanase and a low level of cellulase activity were found in RLC. The level of xylanase activity was consistent with that found by Morrison[16]. From our initial observations, it is not possible to say whether the enzyme is of plant origin or derived from rumen bacteria.

EXPERIMENTAL

General methods. — Total carbohydrate (expressed as glucose) was determined by the phenol-sulphuric acid method[17]. Sugars released by hydrolysis were determined[18] as their alditol acetates by g.l.c. at 225° on a glass column (2 m × 0.4 cm) of 3% SP 2340 on Supelcoport (100–200 mesh). Samples of dried grass and LCC extracted from dried grass were hydrolysed as described by Saemen *et al.*[19]. For rumen complexes, hydrolysis with 2M trifluoroacetic acid for 2 h at 120° was equally effective.

Acetyl groups were determined as described by Bethge and Lindstrom[20], but pivalic acid was used as the standard[18]. Uronic acids were determined by the method of Blumenkrantz and Asboe-Hansen[21]. Hexosamine, reducing sugar, and amino acid were determined as described[22]. Monitoring for protein and polyphenolic material was carried out using Folin's reagent[23].

Preparation of rumen liquor complexes. — Rumen liquor from several cannulated sheep fed on a diet of dried ryegrass was collected about 1 h after feeding and combined to give ~3 L of fluid. Samples were cooled on collection and all subsequent operations were carried out at 4°. After straining through gauze to remove large particles of undigested grass, the fluid was centrifuged (72,000*g* for 30 min), the supernatant solution (~2 L) was dialysed against running water for 48–72 h and freeze-dried to give RLC (~4.5 g).

Preparation of fractions A and B. — A solution of RLC (0.3 g) in 0.2M sodium chloride was applied to a column (85 × 2.6 cm) of Sephadex G50 and eluted with the same solvent. Fractions (8 mL) were assayed for carbohydrate and polyphenol-protein.

Preparation of fraction I and II. — A solution of RLC in water was passed through an Amicon ultrafiltration cell fitted with a PM10 membrane. The low-molecular-weight filtrate (fraction II) was concentrated by rotary evaporation. Each fraction was then freeze-dried.

Lipid-extraction of RLC. — To a suspension of RLC (3 g) in methanol (50 mL) was added chloroform (100 mL), and the mixture was stirred for 1 h at room temperature. The RLC was recovered by centrifugation (2000*g*, 20 min). The extraction was repeated six times and then the RLC was dried *in vacuo*.

Partition with phenol. — A solution of RLC (150 mg) in 0.05M sodium tetraborate buffer (pH 8.0, 20 mL) was partitioned[25] with hot aqueous 90% phenol (20 mL). The clear buffer phase was removed and the insoluble material at the interface was partitioned a further two times, the buffer phases being combined. The phases were concentrated *in vacuo* with repeated additions of water to remove phenol, followed by evaporation with methanol acidified with acetic acid to remove borate. The fractions were then dissolved in water and freeze-dried.

Isolation of LCC from dried grass. — A sample of dried grass (19 g) was milled in a J.K. mill with cooling for 2 min, then extracted with boiling $CHCl_3$–MeOH (2:1, 500 mL) for 30 min, and dried by treatment with alcohol and ether. The

sample was further extracted with 0.05M phosphate buffer (pH 6.0, 500 mL) for 30 min at 60°. A suspension of the dried residue in methyl sulfoxide (400 mL) was stirred for 7 days and then concentrated to dryness, and the residue (101 mg) was dried by successive treatments with ethanol, acetone, and ether. Yield, 101 mg.

Delignification[26] *of RLC fractions.* — Each complex (1 g) was dissolved in water (100 mL) with sonication and the solution was heated to 72° with nitrogen passing over this surface. Five additions of glacial acetic acid (0.5 mL) and sodium chloride (1 g) were made at intervals of 15 min. After a further 15 min, each solution was cooled, nitrogen was passed through for 1 h, and it was then dialysed and freeze-dried.

Proteolytic digestions. — (*a*) *Pronase.* To a solution of each complex (500 mg) in 10mM $CaCl_2$ (50 mL) adjusted to pH 8.0 was added purified[24] Pronase (50 mg), and the mixture was incubated at 37°. The pH was maintained at 8.0. The release of amino acids was monitored using ninhydrin; when complete, the solution was heated for 3 min at 100°. After adjustment of the pH to 6.5–7.0, the solution was diluted with ethanol (4 vol.) and the precipitate was collected and dried. The supernatant solution contained 1–2% of the total carbohydrate.

(*b*) *Pepsin.* A solution of each complex (500 mg) in water (50 mL) was adjusted to pH 2.0, pepsin (250 mg) was added, and the mixture was incubated at 37°. Release of amino acids was monitored as in (*a*); when complete, the solution was heated for 3 min at 100°. Since the pepsin contained a high proportion of lactose, this was removed by gel filtration of the incubation mixture on Sephadex G-50. The fraction containing lignin and carbohydrate was concentrated and freeze-dried. There was quantitative recovery of carbohydrate.

Determination of reducing end groups in complexes. — A solution of each complex (1 g) in water (70 mL) was adjusted to pH 9.0. Sodium borohydride (300 mg) was added and each solution was left for 5 h at room temperature. More (50 mg) sodium borohydride was added, and each solution was left overnight at room temperature. Acetic acid was added to pH 5.0 and each solution was dialysed and freeze-dried.

Samples of the reduced complexes were hydrolysed with 2M trifluoroacetic acid for 2 h at 120° and inositol was added as the internal standard. Each hydrolysate was concentrated to dryness *in vacuo* and to a solution of each residue in 0.1M acetate buffer (pH 4.0, 1.0 mL) were added sulphamic acid (2.5 mg) and sodium chlorite (2 mg). Each solution was left in the dark for 15 days at room temperature, then adjusted to pH 7 with NaOH, and passed through 2–3-mL columns of Dowex-1 (AcO^-), 50 (H^+), and 1 (AcO^-) resins. Aqueous 3% sodium acetate (0.5 mL) was added to each solution before concentration to dryness *in vacuo*. Acetylation was carried out by adding acetic anhydride (1 mL) and heating for 3 h at 120°. After removal of acetic anhydride, each sample was extracted with dichloromethane and the alditol acetates in the extract were analysed by g.l.c.

Determination of enzyme activities. — Glycosidase activities were determined as previously described[27] with the appropriate nitrophenyl glycopyranoside as sub-

strate. Xylanase activity was determined as described by Morrison[16] except that 0.05M dithiothreitol was used in order to diminish the blank absorbance. Carboxymethylcellulase activity was determined by the method of Wood[28].

Methylation. — Samples for methylation were dried over P_2O_5 *in vacuo* and then dispersed in methyl sulfoxide (1.5 mL) with ultrasonic agitation under nitrogen for 1–2 h. Freshly prepared potassium methylsulphinylmethanide (1.5 mL) was added under nitrogen, and the mixture was ultrasonicated for 30 min and then stored overnight at room temperature. Methyl iodide or trideuterimethyl iodide (1 mL) was then added in portions during 1 h with cooling. After standing for a further 1 h, the mixture was poured into saturated aqueous KCl (25 mL), the pH was adjusted to 1.5 with 2M HCl, and the mixture was extracted thrice with chloroform. The combined extracts were washed with 0.05M HCl and water (× 4) and added to quebrachitol (0.506 mg), and the mixture was concentrated to dryness.

Methylated samples were treated with aqueous 90% formic acid (1 mL) for 5 h at 100° under nitrogen. The formic acid was then evaporated under reduced pressure, and the residue was treated with 0.25M H_2SO_4 (1 mL) for 16 h at 100°. The hydrolysate was neutralised with Amberlite IRA-400 (HCO_3^-) resin and concentrated to dryness, and the residue was treated with aqueous sodium borohydride (1 mL, ~ 10 mg) for 3 h at room temperature. The excess of borohydride was decomposed with aqueous 50% acetic acid, and methanol acidified with acetic acid (100:1) was then evaporated (× 4) from the residue followed by methanol. Acetic anhydride (1 mL) was added, the mixture was heated for 2 h at 100° and co-concentrated with toluene, and the residue was partitioned between chloroform and water. The chloroform layer was washed with water (4 ×) and concentrated to dryness. The remaining partially methylated alditol acetates were dried *in vacuo* over P_2O_5.

Reduction of methylated samples. — To a solution of the dried methylated sample (~ 5 mg) in tetrahydrofuran (5 mL) was added lithium aluminium deuteride (~ 10 mg), and the mixture was boiled under reflux for 4 h. Excess of reducing agent was decomposed by addition of ethyl acetate, then methanol, and water. The mixture was neutralised with 2M H_3PO_4, filtered, and concentrated under reduced pressure, the residue was hydrolysed, and the products were derivatised as described above.

G.l.c. — Partially methylated alditol acetates were analysed with a Carlo-Erba Fractovap 4160 gas chromatograph, using on-column injection and flame-ionisation detection[13], a Spectra-physics SP 4100 or a Trivector Trilab III computing integrator, and a Prime 550 computer. Retention coefficients were calculated, and a provisional identification of the peaks was made using algorithms developed from those described previously[13]. The peaks for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and quebrachitol penta-acetate were used for calculation of retention coefficients and quebrachitol was used as an internal standard. Separations were performed on fused-silica columns of SP 1000 (50 m × 0.3 mm) and SIL 10/C (25 m × 0.3 mm). Final identifications were obtained by averaging the results from the two phases when the provisionally identified component was well resolved on both

columns, and by difference, adjusted for overall recovery, when the component co-chromatographed with other component(s) on one or both phases. In general, agreement was better than ±10%. Almost all identifications were confirmed by mass spectrometry.

Preparative g.l.c. — A Carlo-Erba 4100 gas chromatograph equipped with an effluent splitter and fitted with a stainless steel column (2 m × 4 mm) of 3% SP2340 on Gas-chrom Q (100–120 mesh) was used with nitrogen as carrier gas. Effluent fractions were collected in capillary tubes (80 mm × 1 mm ID) manually connected to the effluent stream.

Mass spectrometry. — A VG Micromass 16 mass spectrometer was directly coupled to a Pye 104 gas chromatograph. The above capillary columns were used with a splitless injector.

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